

# Structure and function of tetanus and botulinum neurotoxins

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0. ABSTRACT	423
1. INTRODUCTION	424
2. PRESYNAPTIC ACTIVITY	425
3. STRUCTURE OF TETANUS AND BOTULINUM NEUROTOXINS	429
4. CELL BINDING	434
5. INTERNALIZATION	439
6. TRANSLOCATION INTO THE NEURONAL CYTOSOL	440
7. ZINC-ENDOPEPTIDASE ACTIVITY OF CLOSTRIDIAL NEUROTOXINS	443
8. VAMP, SNAP-25 AND SYNTAXIN	446
9. TARGET RECOGNITION	448
10. THE NEUROEXOCYTOSIS APPARATUS AND THE CLOSTRIDIAL NEUROTOXINS	450
11. WHY THE NEUROTOXINS?	451
12. CONCLUDING REMARKS AND FUTURE PERSPECTIVES	452
13. ACKNOWLEDGEMENTS	453
14. REFERENCES	453

## 0. ABSTRACT

Tetanus and botulinum neurotoxins are produced by *Clostridia* and cause the neuroparalytic syndromes of tetanus and botulism. Tetanus neurotoxin acts mainly at the CNS synapse, while the seven botulinum neurotoxins act peripherally. Clostridial neurotoxins share a similar mechanism of cell intoxication: they block the release of neurotransmitters. They are composed of two disulfide-linked polypeptide chains. The larger subunit is responsible for neurospecific binding and cell penetration. Reduction releases the smaller chain in

the neuronal cytosol, where it displays its zinc-endopeptidase activity specific for protein components of the neuroexocytosis apparatus. Tetanus neurotoxin and botulinum neurotoxins B, D, F and G recognize specifically VAMP/synaptobrevin. This integral protein of the synaptic vesicle membrane is cleaved at single peptide bonds, which differ for each neurotoxin. Botulinum A, and E neurotoxins recognize and cleave specifically SNAP-25, a protein of the presynaptic membrane, at two different sites within the carboxyl-terminus. Botulinum neurotoxin type C cleaves syntaxin, another protein of the nerve plasmalemma. These results indicate that VAMP, SNAP-25 and syntaxin play a central role in neuroexocytosis. These three proteins are conserved from yeast to humans and are essential in a variety of docking and fusion events in every cell. Tetanus and botulinum neurotoxins form a new group of zinc-endopeptidases with characteristic sequence, mode of zinc coordination, mechanism of activation and target recognition. They will be of great value in the unravelling of the mechanisms of exocytosis and endocytosis, as they are in the clinical treatment of dystonias.

## I. INTRODUCTION

Tetanus is a painful and often lethal syndrome, characterized by a spastic paralysis in which opposing skeletal muscles contract spasmodically (van Heyningen, 1968; Bleck, 1989). These acute manifestations often shadow myopathies deriving from neuromuscular junction deficits as well as an overactivity of the autonomic system. Death follows body exhaustion and occurs by respiratory failure or heart collapse. For twenty four centuries tetanus has been considered a neurologic disease until the identification of *Clostridium tetani* (Carle & Rattone, 1884; Kitasato 1889), a bacterium that cause tetanus via the release of a protein toxin, termed tetanus neurotoxin (TeNT) (Faber, 1890; Tizzoni & Cattani, 1890a, b; Kitasato, 1891).

At variance from tetanus, botulism is characterized by a generalized muscular weakness, which first interests ocular and throat muscles and extends later to all skeletal muscles (Sakaguchi, 1983; Smith & Sugiyama, 1988; Hatheway, 1995). In most cases this flaccidity goes unnoticed, and the low evidence of the symptoms accounts for the relatively recent identification of this disease (van Ermengem, 1897; Smith & Sugiyama, 1988). This is also in agreement with the finding of individuals with serum anti BoNT antibodies without a clinical record of botulism (Schiavo *et al.* unpublished work). In the more severe forms, a generalized flaccid paralysis, impairment of respiration and autonomic functions are evident and death results from respiratory failure (Smith & Sugiyama, 1988; Hatheway, 1995). The causative agents of botulism were shown to be neurotoxigenic strains of *Clostridium botulinum* (van Ermengem, 1897), and, more recently, *Cl. barati* and *Cl. butyricum* (Hall *et al.* 1985; Aureli *et al.* 1986). So far, seven different serotypes of botulinum neurotoxin (BoNT), termed A to G, have been identified, while only one form of TeNT is known.

Bacteria of the genus *Clostridium* are strictly anaerobic and are widely distributed in a variety of environments, mainly in the form of spores. Spores of

*Cl. tetani* may contaminate even minor breaks of the skin and, under appropriate conditions, they germinate. At the present time, the most common way of infection in humans is the cut of the umbilical cord under non sterile conditions, which leads to the deadly form of *tetanus neonatorum*. The WHO estimated that more than 450,000 infants died of such a disease in the world in 1991 (Whitman *et al.* 1992). After a moderate proliferation without spreading and with no relevant signs of inflammation, these bacteria begin to produce and accumulate in the cytosol tetanus neurotoxin and tetanolysin, a cholesterol binding cytolysin. A neurotoxin may reach as much as 10% of the total bacterial protein mass before it is released by cell autolysis (Bonventre & Kempe, 1960). All the clinical symptoms of tetanus are caused by TeNT. The role of tetanolysin, if any, in the pathogenesis of tetanus is unknown (Mitsui *et al.* 1980; Blumenthal & Habig, 1984). It is possible that it contributes to create a zone of necrosis in the wound, around the site of spore germination, thus allowing for a better growth of *Cl. tetani*.

Very rarely, botulism follows wound infection with spores of *Cl. botulinum* (wound botulism). The most common source of intoxication is with foods, contaminated by spores of *Cl. botulinum* and preserved under anaerobic conditions that favour germination, proliferation and production of BoNT (Sakaguchi, 1983; Smith & Sugiyama, 1988; Hatheway, 1995). All BoNT serotypes are sensitive to low pH and proteases of the gastric juice. To protect them during their passage through the stomach, BoNTs are produced as complexes with other proteins. The complexes that survive this step, dissociate at the higher pH of the intestinal lumen and BoNT is adsorbed through the epithelial layer and enters the general circulation. Hence, tetanus and botulism are intoxications, rather than infections. The only exception is infant botulism, a consequence of ingestion of clostridial spores and colonization of the intestine of the newborn, which lacks the normal flora and thus allows germination and proliferation of the bacterium (Arnon, 1980; Hatheway, 1995).

As a consequence of the fact that a single protein is responsible for all the clinical symptoms of tetanus and botulism, these diseases can be completely prevented by anti toxin specific antibodies (Middlebrook & Brown, 1995). Toxin neutralizing antibodies can be acquired passively by injection of antibodies isolated from immunized donors or, actively, as a result of vaccination with toxoids. Toxoids are toxins transformed chemically or genetically in such a way as to be atoxic, but still immunogenic (Ramon & Descombez, 1925). Together with diphtheria vaccine, tetanus vaccine is the major product of the biotechnology industry. Vaccination against botulism is performed only on people involved in manipulation of BoNTs, since severe botulism is rather rare in the more developed countries.

## 2. PRESYNAPTIC ACTIVITY

TeNT and BoNT's are the most potent toxins known. In the mouse, the 50% lethal dose is comprised between 0.1 ng and 1 ng of toxin per Kg of body weight. Such figures are probably much lower in the wilderness, where even a very small

deficit in mobility may be sufficient to prevent survival. Different animal species show a great range of sensitivity to TeNT and to the different BoNTs. Humans and horses are as sensitive to these neurotoxins as mice, or more, while rats and birds are rather resistant to TeNT, and turtles are insensitive (Payling-Wright, 1955).

This terrible toxicity of the clostridial neurotoxins (CNTs) derives from their absolute neurospecificity as well as from their catalytic activity. TeNT and BoNTs bind very specifically to the presynaptic terminal of the neuromuscular junction of motoneurons (NMJ). CNTs are also able to bind to synaptic terminals of sensory and adrenergic neurons. Neither the receptor(s) on the presynaptic membrane, nor the binding constants are known. However, binding affinity must be very high since deadly doses in the mouse are such to give toxin concentrations in the body fluids well below picomolar. From the presynaptic membrane, the BoNTs gain access to the neuronal cytosol and block the release of acetylcholine (ACh), thus causing a flaccid paralysis (Burgen *et al.* 1949; Mellanby, 1984; Simpson, 1989). Also TeNT binds to the motoneuron presynaptic membrane, but an analysis of clinical symptoms indicates that its action at this peripheric level is nihil or very limited. TeNT is transported retrogradely inside the motoneuron axon up to the spinal cord (Bruschettini, 1892; Erdmann *et al.* 1975a; Price *et al.* 1975; Stockel *et al.* 1975; Vallee & Bloom, 1991). An intra-axonal ascent transport rate of 7.5 mm/h has been estimated by Stockel *et al.* (1975), but neuromuscular stimulation enhances this rate (Ponomarev, 1928; Kryzhanovsky, 1958; Hughes & Whaler, 1962; Wellhoner *et al.* 1973; Habermann *et al.* 1980). Habermann and Dimpfel (1973) reported that, from the spinal cord, TeNT could reach the brain stem, but not the forebrain nor the cerebellum. The retrograde intra-axonal transport of TeNT is now so well established that the determination of the amount of  $^{125}\text{I}$ -TeNT which reaches the spinal cord is used as an assay of the peripheral neurodegenerative potency of drugs and chemicals (Sahenk & Mendell, 1981; Moretto *et al.* 1987).

Once in the spinal cord, TeNT migrates trans-synaptically into the inhibitory interneurons of the spinal cord (Schwab & Thoenen, 1976; Schwab *et al.* 1979). TeNT is released from the motoneuron into the synaptic cleft with the inhibitory interneuron and enters the cytosol of this latter cell, where it blocks the release of inhibitory neurotransmitters (Brooks *et al.* 1955, 1957; Curtis *et al.* 1976; Benecke *et al.* 1977; Bergey *et al.* 1983). At early stages, TeNT does not act directly on excitatory synapses in the CNS (Brooks *et al.* 1955, 1957; Mellanby & Green, 1981; Wellhoner, 1982, 1992; Bergey *et al.* 1983; Williamson *et al.* 1992), but it may do so at later stages (Takano *et al.* 1983). Excitatory synapses of spinal cord neurons in culture are affected by TeNT (Bergey *et al.* 1983; Williamson *et al.* 1992). During trans-synaptic migration, TeNT can be neutralized by anti-toxin antibodies injected in the spinal fluid (Erdmann *et al.* 1981). The amount of toxin that reaches the CNS, after uptake at the PNS, is clearly an important parameter that determines the severity of the disease and contributes to account for the different toxicity of TeNT in different vertebrates (Payling-Wright, 1955). The limited data available (Weller *et al.* 1986; Habermann & Weller, 1989) indicate

that TeNT in the very small intersynaptic space between motoneuron and inhibitory interneuron may reach a concentration much higher than that present at the NMJ.

The blockade of inhibitory synapses at the spinal cord impairs the neuronal circuit that ensures balanced voluntary muscle contraction, thus causing the spastic paralysis characteristic of tetanus (Mellanby & Green, 1981; Wellhoner, 1982, 1992; Simpson, 1989). After its release from alpha-motoneurons, TeNT may spread locally to affect also inhibitory pathways to gamma motoneurons and to increase sympathetic activity by releasing inhibition from the sympathetic system (Wellhoner, 1982). The half-life of  $^{125}\text{I}$ -TeNT in the rat spinal cord has been estimated to be around 6.5 days (Habermann & Dimpfel, 1973), a figure comparable to that found in cells in culture (Habig *et al.* 1986; Marxen & Bigalke, 1991).

Hence, the opposite clinical symptoms of tetanus and botulism result from different sites of action of TeNT and BoNTs, rather than from a different mechanism of action. This neat distinction between the central site of activity of TeNT and peripheral site of action of the BoNTs exists only at clinical concentrations, i.e.  $< 10^{-12}$  M. To obtain rapidly relevant effects, hundreds of mouse lethal doses are frequently used. This is obligatory choice in experiments with TeNT insensitive species such as birds or fishes or *in vitro* with cultured cells or with the rodent hemi-diaphragm preparation: under such conditions TeNT also affects peripheral synapses (reviewed in Mellanby & Green, 1984; Habermann & Dreyer, 1986; Wellhoner, 1992). In any case, the only known action of TeNT and BoNTs is presynaptic and consists in a persistent inhibition of the release of neurotransmitters, including glycine, GABA, ACh, D-Asp, glutamate and norepinephrine; moreover the release of opioids, oxytocin and vasopressin is also inhibited (reviewed in Wellhoner, 1992). If the nerve specific binding and uptake processes are by-passed, the action of CNTs can be extended to a variety of non-neuronal cells. When injected inside cells or added to permeabilized cells, the CNTs can inhibit many, but not all, exocytotic events in a wide range of cells (Penner *et al.* 1986; Bittner & Holz, 1988; Ahnert-Hilger *et al.* 1989a, b; Bittner *et al.* 1989a, b; Stecher *et al.* 1989; McInnes & Dolly, 1990; Dayanithi *et al.* 1992, 1994; Ikonen *et al.* 1995; Sadoul *et al.* 1995; Regazzi *et al.* 1995). At high doses BoNT/D inhibits exocytosis in bovine adrenal cells (Knight *et al.* 1985).

Careful electrophysiological studies can be performed at the vertebrate NMJ and this is the only synapse where TeNT and several BoNT types have been consistently tested and compared (Molgo *et al.* 1990; van der Kloot & Molgo, 1994; Poulain *et al.* 1995), following the milestone work of Burgen *et al.* (1949). Transmission of a nerve muscle impulse follows a presynaptic depolarization that causes the opening of voltage-gated calcium channels. This leads to a very rapid local increase of calcium up to 200–300  $\mu\text{Molar}$ , which triggers, within 2–300 microseconds, the fusion of small synaptic vesicles (SSVs) bound to specialized ‘active’ zones of the presynaptic membrane (Katz, 1966; Llinas, 1992; Burgoyne & Morgan, 1993; Almers, 1994; Fesce *et al.* 1994; Monk & Fernandez, 1994; van der Kloot & Molgo, 1994). The synchronous release of these ACh quanta causes

a large post-synaptic depolarization, termed end plate potential (EPP). The resting NMJ spontaneously releases quanta of ACh, each of which is contained in a single small synaptic vesicle (SSV) of 50 nm diameter. This release causes a post-synaptic depolarization, termed miniature endplate potential (MEPP) (Katz, 1966). Occasionally, giant MEPPs can be observed. They account for 1–3 % of the total number of synaptic events and correspond to a large calcium-independent discharge of vesicles, since the amount of ACh released is sufficient to activate the muscle fibre (Liley, 1957; Kim *et al.* 1984; Thesleff, 1986; Thesleff *et al.* 1990). Giant MEPPs have been suggested to derive from the release of ACh contained in endosomal compartments precursors of the SSV (Bauerfeind *et al.* 1994) or as a result of repair processes at damaged neuronal terminals (Thesleff *et al.* 1990; Poulain *et al.* 1995). After release, the SSVs undergo rapid re-uptake in a dynamin-dependent process and are refilled with neurotransmitter by proton-driven neurotransmitter transporters (Sudhof & Jahn, 1991; Kelly, 1993; Bennett & Scheller, 1994; Robinson *et al.*, 1994; von Gersdoff & Matthews, 1994; Takei *et al.* 1995; Schuldtner *et al.* 1995).

TeNT and all the BoNTs cause a large and persistent blockade of EPPs as well as a large reduction of the frequency of MEPPs, with no change of the size of each quantum (Harris & Miledi, 1971; Kryzhanovsky *et al.* 1971; Mellanby & Thompson, 1972; Duchen & Tonge, 1973; Boroff *et al.* 1974; Cull-Candy *et al.* 1976; Habermann *et al.* 1980; Dreyer & Schmitt, 1981, 1983; Kanda & Takano, 1983; Sellin *et al.* 1983; Bevan & Wendon, 1984; Bigalke *et al.* 1985; Kaufmann *et al.* 1985; Bergey *et al.* 1987; Dolly *et al.* 1987; Gansel *et al.* 1987; Poulain *et al.* 1988; Mochida *et al.* 1989; Molgo *et al.* 1989*a, b*). Hence, TeNT and BoNTs greatly decrease the number of vesicles capable of fusion and release, but do not alter their neurotransmitter content, nor they interfere with the processes of neurotransmitter synthesis, uptake and storage (reviewed by Gundersen, 1980). Similarly, TeNT and BoNTs do not affect the propagation of the nerve impulse nor calcium entry and disposal at the synaptic terminal (Gundersen *et al.* 1982; Dreyer *et al.* 1983; Mallart *et al.* 1989; Molgo *et al.* 1989*a*). In contrast to the effect of TeNT and BoNTs on EPPs and MEPPs, the frequency of giant MEPPs is not altered or even increases in the intoxicated NMJ (Sellin *et al.* 1983; Kim *et al.* 1984; Thesleff, 1986; Molgo *et al.* 1990). Careful morphological examinations of poisoned NMJs have shown no evident structural changes, except for an increase in the number of SSVs present at the active zones of synaptic terminals (Kryzhanovsky *et al.* 1971; Podzdnyakov *et al.* 1972; Duchen, 1973 *a, b*; Mellanby *et al.* 1988; Neale *et al.* 1989; Pecot-Dechavassine *et al.* 1991; Hunt *et al.* 1994).

Within this general picture of close similarity in the final result, the eight CNTs show remarkable qualitative differences in their presynaptic action. While the evoked ACh release remains synchronous in the BoNT/A and /E poisoned NMJ, TeNT and BoNT/B, /D and /F cause a desynchronization of the quanta released after depolarization (Harris & Miledi, 1971; Dreyer & Schmitt, 1983; Bevan & Wendon, 1984; Gansel *et al.* 1987; Molgo *et al.* 1989*a*). Aminopyridines raise the synaptic calcium level by inhibiting potassium channels. When added to poisoned

NMJs, the evoked neurotransmitter release remains largely asynchronous in NMJ treated with TeNT and BoNT/B, /D and /F, but not in those incubated with BoNT/A and /E (reviewed by: Sellin, 1987; Molgo *et al.* 1990; Wellhoner, 1992; Poulain *et al.* 1995). The calcium ionophore A23187, added to the intoxicated mouse diaphragm, partially reverses the effect of BoNT/A, is poorly effective on BoNT/E and has no effect on TeNT treated preparations (Ashton *et al.* 1993). Thus, an increase in synaptic calcium level antagonizes the effect of BoNT/A and /E, but not that of TeNT, BoNT/B, /D and /F. A difference in the mechanism of action of the CNTs is also suggested by double poisoning the NMJ with a clostridial neurotoxin followed by  $\alpha$ -latrotoxin (LT). LT is the major toxic component of the venom of the black widow spider and causes a massive depletion of SSV from synaptic terminals (Valtorta *et al.* 1984; Meldolesi *et al.* 1986; Petrenko *et al.* 1991). LT counteracts the action of BoNT/A, but not that of TeNT or BoNT/B (Gansel *et al.* 1987). These data indicate that the neurotoxins have different targets within the presynaptic terminal and this conclusion is now fully substantiated by the identification of the molecular targets of each CNT (see below sections 8 and 9).

A fully paralysed NMJ loses its functionality, but the motoneuron remains alive as well as the innervated muscle fibre, which undergoes a transient atrophy (Duchen & Tonge, 1973). New synaptic terminals outgrow from the axon near the damaged NMJ within few weeks. This sprouting leads to the generation of new functional nerve-muscle contacts and the muscle fibre regains its pre-poisoning size (reviewed in Thesleff, 1989). The demonstration that the inhibition of the nerve-muscle impulse is followed by a functional recovery of the NMJ has lead to the therapeutic use of these neurotoxins in dystonias and strabismus (Scott, 1989; Jankovic & Hallett, 1994). Injection of minute amounts of BoNT into the muscle(s) to be paralyzed leads to a depression of the symptoms lasting few months. BoNT/A is generally used, but other BoNT types are currently under clinical trial. This is now recognized as the best available treatment for dystonias and for certain types of strabismus. It can be repeated several times, without major side effects such as the development of an immune response. In this latter case, treatment can be continued with another BoNT serotype (Jankovic & Hallett, 1994). TeNT has been used as a epileptogenic agent and in the induction of selective neuronal degeneration (Brace *et al.* 1985; Bagetta *et al.* 1991).

### 3. STRUCTURE OF TETANUS AND BOTULINUM NEUROTOXINS

The similar mechanism of action of the eight CNTs is paralleled by a common structural organization. As depicted in Fig. 1, CNTs are produced as inactive polypeptide chains of 150 kDa without a leader sequence. After release by bacterial lysis, the clostridial neurotoxins are cleaved by several bacterial and tissue proteinases at an exposed protease-sensitive loop. An active di-chain neurotoxin is thus generated (DasGupta, 1989, 1994; Weller *et al.* 1989; Kriegstein *et al.* 1991). The heavy chain (H, 100 kDa) and the light chain (L, 50 kDa) are bridged by a single interchain disulfide bond essential for

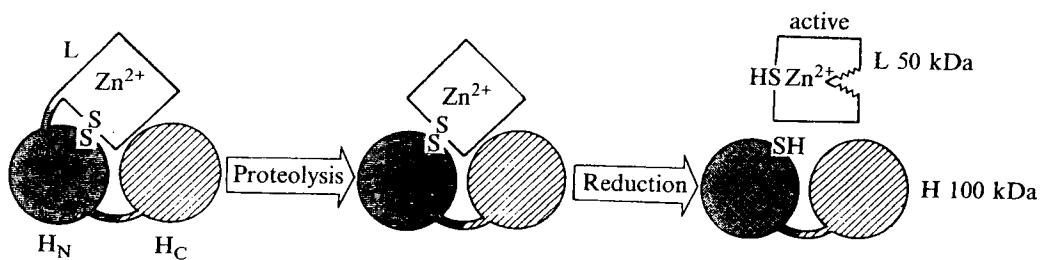


Fig. 1. Scheme of the structure and mechanism of activation of tetanus and botulinum neurotoxins. The toxins are produced as an inactive single polypeptide chain of 150 kDa, composed of three 50 kDa domains, connected by protease-sensitive loops. The toxins are activated upon selective proteolytic cleavage which generates two disulfide-linked chains: L (50 kDa) and H (100 kDa). The three domains play different functional roles in cell penetration: H<sub>C</sub> is responsible for cell binding and H<sub>N</sub> for cell penetration. Reduction takes place inside the nerve cells and liberates the activity of the L chain in the cytosol. L is a zinc-endopeptidase specific for protein components of the neuroexocytosis apparatus.

neurotoxicity of the CNT added extracellularly (Schiavo *et al.* 1990; de Paiva *et al.* 1993a). There may be very little protein-protein interactions between the two chains since, after reduction, they can be separated with diluted urea solutions (Matsuda & Yoneda, 1975; Sathyamoorthy & DasGupta, 1985; Weller *et al.* 1989). BoNT/A and the COOH-terminal TeNT domain have been recently crystallized (Stevens *et al.* 1991; Anderson *et al.* 1993). Analysis of secondary structure of TeNT and BoNT/A and /E with different spectroscopic methods indicates contents of  $\alpha$ -helix and  $\beta$ -sheets consistent with those of most globular proteins (Robinson *et al.* 1981; Singh *et al.* 1990a, b; De Filippis *et al.* 1995). Biochemical and low resolution electron microscopic studies (Robinson *et al.* 1988), as well as comparison with the known structure of other bacterial toxins which penetrate cells similarly to TeNT and BoNT (Menestrina *et al.* 1994; Montecucco *et al.* 1994), lead to the proposal that TeNT and BoNTs are folded into three distinct 50 kDa domains (Schiavo *et al.* 1993a; Montecucco & Schiavo, 1993), as depicted in Fig. 1. This structural organization is functionally related to the fact that these toxins act inside cells. Their cell intoxication mechanism consists of four distinct steps: (1) binding, (2) internalization, (3) membrane translocation and (4) enzymatic target modification (Montecucco *et al.* 1994). The L chain is responsible for the intracellular catalytic activity. The amino-terminal 50 kDa domain of the H chain (H<sub>N</sub>) is implicated in membrane translocation, while the carboxy-terminal part (H<sub>C</sub>) is mainly responsible for the neurospecific binding.

The amino acid sequence of all eight clostridial neurotoxins has been derived from the corresponding genes (for reviews and references see: Niemann, 1991; Minton, 1995). The L chains and H chains are composed on average of 439 residues and 843 residues, respectively. They present homologous segments separated by regions of none or little similarity. The most conserved portions of the L chains are the amino-terminal (one hundred residues) and central regions (residues 216–244, numbering of TeNT), as well as the two cysteines forming the

Table 1.  $^{65}\text{Zn}^{2+}$  binding to TeNT and BoNT/A, /B and /E

Toxin type	$K_D$ (nM)	$n$	$K_D$ ( $\mu\text{M}$ )	$n$
TeNT	100	0.8	2.2	1.9
BoNT/A	70	1.0	1.3	2.7
BoNT/B	95	0.9	1.9	2.3
BoNT/E	105	1.0	1.9	2.8

interchain disulfide bond. Eight amino-terminal residues and sixty-five carboxy-terminal residues can be deleted from TeNT without loss of activity (Kurazono *et al.* 1992). The 216–244 region contains the His-Glu-Xaa-Xaa-His binding motif of zinc-endopeptidases (Jongenel *et al.* 1989; Vallee & Auld, 1990; Jiang & Bond, 1992; Schiavo *et al.* 1992*a, b*; Wright *et al.* 1992). This observation led to the demonstration that CNTs are zinc-proteins (Schiavo *et al.* 1992*a, b*, 1993*b*, 1994, 1995*a*; Wright *et al.* 1992; Yamasaki *et al.* 1994*a*). Due to the limited amount of toxins available, zinc content of purified preparations of CNTs has been determined directly only on TeNT and on BoNT/A, /B, /C, /E and /F. One atom of zinc is bound to the L chain TeNT, BoNT/A, /B, and /F (Schiavo *et al.* 1992*a, b*; 1993*b*). The results of a  $^{65}\text{Zn}^{2+}$  flow dialysis estimation of the affinity of zinc binding to TeNT and BoNT/A, /B and /E are summarized in Table 1. These CNTs show a single zinc binding site with  $K_D$  in the 50–100 nMolar range, at the lower limit of the known range of affinities among metallo-proteases, and multiple zinc binding sites with lower affinity (Schiavo *et al.* 1992*b*; Wright *et al.* 1992). BoNT/C binds two atoms of zinc (Schiavo *et al.* 1995*a*). This feature of BoNT/C is shared by neutrophil collagenase, whose three-dimensional structure has been recently determined (Bode *et al.* 1994; Lovejoy *et al.* 1994). One atom of zinc is present at the active site of this metallo-proteinase and it is exchangeable, while the second one is very strongly bound and is thought to play a structural role.

Zinc can be removed from the CNTs with heavy metal chelators, thus generating an inactive apo-neurotoxin. The metal atom can be reacquired upon incubation in zinc-containing buffers to reform the active holo-toxin (Schiavo *et al.* 1992*a, b*, 1993*b*, 1994, 1995*a*; Simpson *et al.* 1993; Hohne-Zell *et al.* 1994).

The zinc atom of zinc-endopeptidases is coordinated either by two or three histidines (Jiang & Bond, 1992; Bode *et al.* 1993). In thermolysin-like enzymes the zinc atom is at a center of a tetrahedron formed by the imidazole rings of the two histidines of the motif, a water molecule bound to the glutamic acid of the motif and the carboxylate of another glutamate residue (Matthews *et al.* 1972; Paupert *et al.* 1988; Thayer *et al.* 1991). At variance, astacin penta-coordinates zinc via three histidines and one tyrosine, whereas adamalysin, the alkaline protease of *Pseudomonas aeruginosa* and collagenase adopt a tetrahedral coordination via three histidines and the glutamate-bound water molecule (Bode *et al.* 1992, 1993, 1994; Baumann *et al.* 1993; Gomis-Ruth *et al.* 1993; Lovejoy *et al.* 1994). The active site

has a cleft shape with the zinc atom in the center and the residues of the motif organized as an  $\alpha$ -helix (Fig. 2). Orientation and volume of the lateral chains of residues at the active site determine the peptide bond specificity. The water molecule bound to the glutamate of the motif is involved in peptide bond hydrolysis with a mechanism that has been studied in detail only for thermolysin (Matthews, 1988).

To determine the number of histidines involved in zinc coordination, the L chains of TeNT and BoNT/A, /B and /E were modified with DEPC, a histidine specific reagent. In each case, two additional histidines were modified in the apo-with respect to the holo-neurotoxin (Schiavo *et al.* 1992a, b), as it is found with thermolysin, but not with astacin and adamalysin (Tonello *et al.* unpublished results). Mutation of Glu-234 of TeNT led to complete loss of proteolytic activity (Li *et al.* 1994; Yamasaki *et al.* 1994a). These results indicate that the zinc atom of CNTs is coordinated via two histidines and a Glu-bound water molecule, as in thermolysin. TeNT mutants at the two histidines of the motif are inactive and bind  $^{65}\text{Zn}^{2+}$  poorly (Yamasaki *et al.* 1994a). Also mutation of the conserved Glu<sup>271</sup>–Glu<sup>272</sup> couple of residues of TeNT, predicted to be in a helical segment (Lebeda & Olson, 1994), results in decreased zinc binding and loss of activity. On this basis, it was suggested that CNTs are thermolysin-like proteases and that one of these two Glu residues is the fourth zinc ligand (Yamasaki *et al.* 1994a). However, the mutational approach to the study of a protein active site in the absence of a structural basis is always biased by the fact that many residues are required for the proper folding and structural stability of a protein. Mutation of such residues leads to the same final result: loss of activity, though they are not directly involved in catalysis (Creighton, 1992). This may be the case here since the two TeNT-corresponding Glu residues of collagenase are located in a segment distant from the active site.

The metal atom of TeNT can be exchanged with other transition metals of similar size with preservation of toxic activity (Höhne-Zell *et al.* 1993; Tonello *et al.* in preparation). The cobalt-substituted TeNT shows an absorption spectrum more similar to that of Co-thermolysin than to that of Co-astacin (Tonello *et al.* in preparation). A comparison of the EXAFS spectra of TeNT, astacin, alkaline protease and thermolysin shows a close similarity of TeNT with astacin and alkaline protease (Morante *et al.* 1995). This result indicates that one (or possibly two) additional aromatic residue(s) is present around the zinc atom of TeNT, in addition to the two histidines. Sequence comparison (Fig. 2) indicates that a Tyr residue (Tyr-243 in TeNT), conserved among all CNTs, is located in the same position as the third zinc ligand histidine of astacin and astacin-like proteinases. Moreover, the replacement of Tyr-243 with Phe in TeNT leads to a great loss of activity, while replacement of the third conserved His preserves activity (Yamasaki *et al.* 1994a). Taken together, these results and considerations suggest that in TeNT the polypeptide chain bends after the first two histidines of the motif to bring the following conserved Tyr, close to the active site zinc, as depicted in Fig. 2. If definitively proven, this would be a novel mode of zinc coordination among metalloproteinases.

BoNT/A	D P A V T L A H E L I H A G H R L Y G
BoNT/A Infant	D P A V T L A H E L I H A E H R L Y G
BoNT/B Gp I	D P A L I L M H E L I H V L H G L Y G
BoNT/B Gp II	D P A L I L M H E L I H V L H G L Y G
BoNT/C	D P I L I L M H E L N H A M H N L Y G
BoNT/D	D P V I A L M H E L T H S L H Q L Y G
BoNT/E	D P A L T L M H E L I H S L H G L Y G
BoNT/E Butyricum	D P A L T L M H E L I H S L H G L Y G
BoNT/F	D P A I S L A H E L I H A L H G L Y G
BoNT/F Barati	D P A I S L A H E L I H V L H G L Y G
BoNT/F Langeland	D P A I S L A H E L I H A L H G L Y G
BoNT/G	D P A L T L M H E L I H V L H G L Y G
TeNT	D P A L L L M H E L I H V L H G L Y G
Zincins	* * * * * * * * * *
Metzincins	H E x x H * * * * * x h x H E x h H x h G h x H

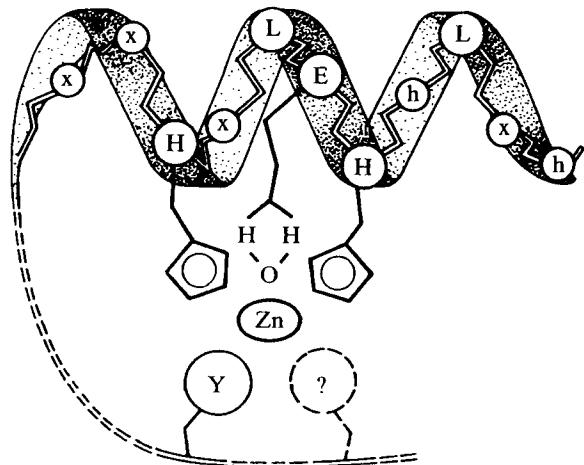


Fig. 2. Zinc coordination by the L chain of clostridial neurotoxin. The upper panel reports the sequence of the longest conserved segment among the L chains of tetanus and botulinum neurotoxins, which is located in the central part of the chain. It contains the zinc-binding motif of metallo-proteinases (zincins) and it is dissimilar from the consensus sequence of the metzincins metallo-proteinase group. The lower panel shows the mode of zinc coordination at the active site of clostridial neurotoxins, as suggested by available data. Whereas the involvement of the two imidazole groups and the water molecule is well established, the involvement of the conserved Tyr present in the segment and of a possible additional aromatic residue is still to be definitively proven. Notice that the suggested Tyr fourth ligand of clostridial neurotoxins occupies the same positions as the His fourth zinc ligand of metzincins.

The L chain of TeNT shows secondary structure contents (27%  $\alpha$ -helix and 43%  $\beta$ -sheet, as determined by CD), consistent with a globular structure (De Filippis *et al.* 1995). There is a substantial agreement between these experimental results and refined structural predictions of the L chain of NTs (Lebeda & Olson, 1994; De Filippis *et al.* 1995). The conserved Trp residue (Trp-43 in TeNT) is buried in a rather rigid environment distant from the zinc atom, which does not appear to contribute significantly to the structural stability of the protein (De Filippis *et al.* 1995).

The H chains are less conserved than the L chains and the carboxyl-terminal

part of Hc (residues 1140–1315 of TeNT) is the most dissimilar (Minton, 1995). This is consistent with the notions that the Hc domain is involved in binding to the nerve terminals and that the different neurotoxins bind to different receptors (see below). On this basis it may be suggested that the protein receptor binding region of TeNT and BoNTs is mainly located within the 180 COOH-terminal residues. A limited degree of similarity of portions of the H chains is also in keeping with the finding that the majority of the serotype specific antibodies recognize epitopes of the H chains. The H<sub>N</sub> domain and the first half of the H<sub>C</sub> domain have stretches, predicted to fold into amphipathic  $\alpha$ -helices (Beise *et al.*, 1994; Lebeda & Olson, 1994). They could be involved in the process of membrane translocation of the L domain in the neuron cytosol (see below).

Comparison of the nucleotide and amino acid sequences of the CNTs clearly indicates that they derive from a common ancestor gene. In this respect, it is significant that the CNT genes are located on genetic mobile elements (Minton, 1995). Spreading of these genes among bacteria of the *Clostridium* genus may take place by bacteriophages or conjugation plasmids or conjugation transposons. Mutation and differentiation of CNT genes is apparent from the fact that variants of the seven BoNTs, with different sequence and similar serotyping, are being detected with the modern molecular genetics methods from different *Clostridia* (Minton, 1995). Moreover, strains that harbour more than one BoNT gene have been identified (Sakaguchi, 1983; Eklund *et al.* 1989).

#### 4. CELL BINDING

After diffusion in the body fluids from the site of production or adsorption, BoNTs and TeNT bind to the presynaptic membrane of the NMJ of alpha-motoneurons. TeNT is also capable of binding to sympathetic and adrenergic fibers (reviewed by Wellhoner, 1992 and by Halpern & Neale, 1995). *In vitro*, CNTs bind to a variety of non neuronal cells, but they do so at concentrations far exceeding those of clinical significance. Few hundreds of binding sites per square  $\mu\text{m}$  appear to be present at rat NMJ for BoNT/A and /B (Black & Dolly, 1986a), whereas the number of TeNT receptors in a neuroblastoma-glioma cell line is around 450 (Wellhoner & Neville, 1987). Despite many efforts spanning almost a century, the chemical nature of the molecule(s) responsible for the neurospecific high affinity binding of the NT to the presynaptic terminal has not been identified.

Following the seminal work of van Heyningen (1959, 1968, 1974), a large number of studies have been devoted to assessing the role of polysialogangliosides in neurotoxin binding (reviewed by Mellanby & Green, 1981; Montecucco, 1986, 1989; Wellhoner, 1992; Halpern & Neale, 1995). The outer membrane layer of the presynaptic membrane contains a large proportion of polysialogangliosides. This provides a large number of acceptors sites for the clostridial neurotoxins that are known to adsorb onto such negatively charged membrane surfaces (Simpson & Rapport, 1971; Zimmermann & Piffaretti, 1977; Holmgren *et al.* 1980; Kitamura *et al.* 1980; Kamata *et al.*, 1986; Ochanda 1986; Montecucco *et al.* 1988, 1989; Walton *et al.* 1988; Marxen *et al.* 1989; Schengrund *et al.* 1991; Schiavo *et al.*

1991a; Halpern & Loftus, 1993). Incubation of cultured chromaffin cells with polysialogangliosides increases their sensitivity to TeNT and BoNT/A (Marzen *et al.* 1989). Preincubation of these neurotoxins with ganglioside mixtures reduces TeNT toxicity and intra-axonal transport (Mellanby *et al.* 1968; Stockel *et al.* 1977). Moreover, treatment of membranes with neuraminidase, which removes the negatively charged sialic acid residues, decreases toxin binding (Williams *et al.* 1983; Yavin & Habig, 1984; Critchley *et al.* 1985; Habermann & Albus, 1986; Bigalke *et al.* 1986; Evans *et al.* 1986; Kitamura & Sone, 1987; Walton *et al.* 1988; Wadsworth *et al.* 1990). Though these studies have clearly established that polysialogangliosides do interact with the various CNTs, no clear demonstration of their direct involvement in the neurospecific binding of these toxins *in vivo* has been provided. As discussed in details elsewhere (Mellanby & Green, 1981; Montecucco, 1986, 1989; Niemann, 1991; Halpern & Neale, 1995) it is very unlikely that polysialogangliosides are the sole receptors of the CNTs at synaptic terminals. An analysis of the literature (references in Mellanby & Green, 1981; Montecucco, 1986, 1989; Wellhoner, 1992; Halpern & Neale, 1995) leads to the following points: (a) the plasmalemma polysialoganglioside content does not correlate with TeNT and BoNT binding and with the differential sensitivity to CNTs of NMJ preparations or cells in culture; (b) polysialogangliosides are not uniquely present in the nervous tissue; (c) TeNT and BoNTs do not show a stoichiometric specific binding for known gangliosides, nor do they bind the oligosaccharide moiety; (d) gangliosides are present in a very large excess with respect to the toxin: the  $G_{D1b}/TeNT$  ratio in the mouse exceeds  $10^9$  (Mellanby & Green, 1981); (e) both *in vivo* (see section 2) and in cultured cells *in vitro*, the eight CNTs show a marked difference in their activity towards different neuronal cells; (f) TeNT and BoNTs bind the same NMJ presynaptic membrane and yet they have a totally different intracellular destiny: the seven BoNTs act at the NMJ, while TeNT migrates to the CNS; (g) TeNT and some of the seven BoNTs do not compete with each other for binding to neuronal membranes.

On the other hand, experiments with cells in culture indicate that proteins of the cell surface may be involved in toxin binding (Pierce *et al.* 1986; Yavin & Nathan, 1986; Parton *et al.* 1988). Moreover, in a NGF-differentiated sub-clone of PC-12 cells, a protein with an apparent molecular weight of 20 kDa was shown to interact with the H chain of TeNT (Schiavo *et al.* 1991b). To account for these results a 'double receptor' mode of binding of clostridial neurotoxins to nerve cells was put forward (Montecucco, 1986, 1989). As depicted in Fig. 3, the neurotoxin is proposed to interact first with negatively charged lipids of the presynaptic membrane via a high capacity interaction(s). Such a mode of binding would constitute a large 'trapping device' for the minute amounts of neurotoxins sufficient to cause the disease. The toxin then moves laterally on the presynaptic membrane surface to bind a protein receptor that is responsible for neurospecificity and uptake at the NMJ. Such encounter of each neurotoxin with its protein receptor is greatly favoured by the bi-dimensionality of the membrane, which greatly reduces the volume of reaction and increases the rate of binding. A high affinity binding is thus obtained, because the toxin-cell association constant

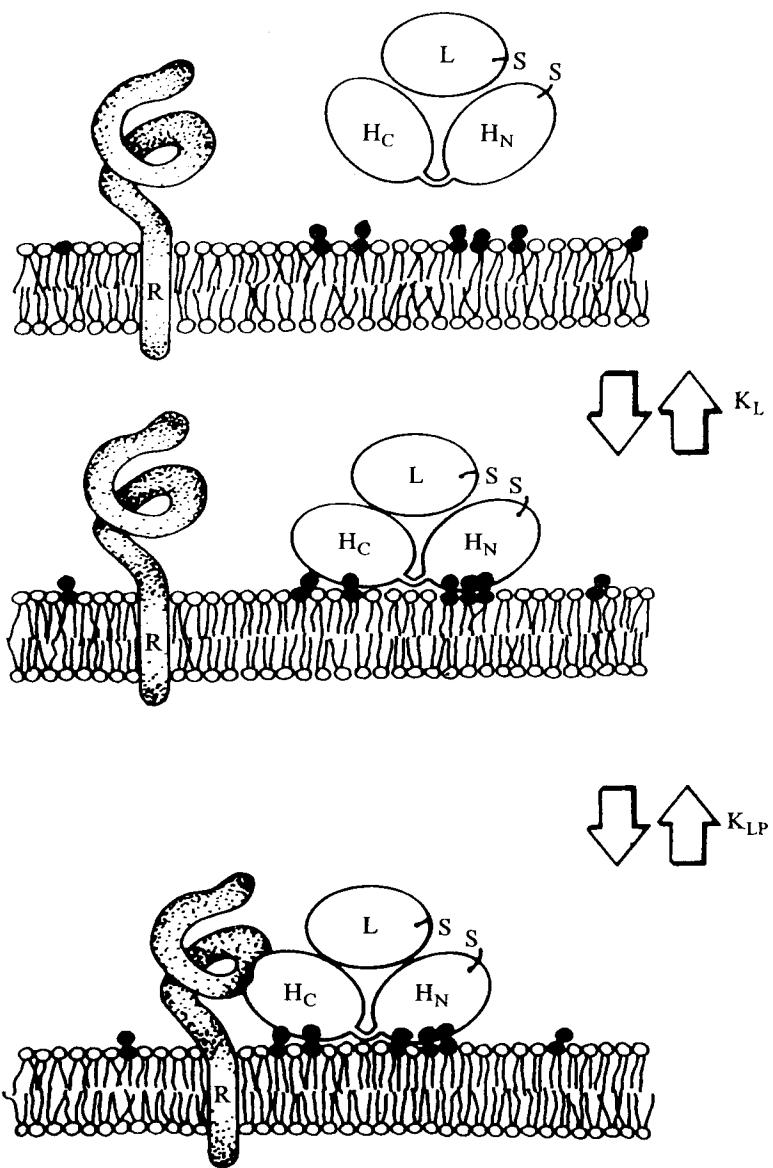


Fig. 3. A double receptor binding of clostridial neurotoxins to the presynaptic terminal plasma membrane. The neurotoxin binds via its H chain to negatively charged lipids of the presynaptic membrane with an affinity constant  $K_L$ . When adsorbed on the membrane surface, the toxin can move laterally and bind via the carboxyl-terminal domain to a protein receptor R. A high affinity binding constant  $K_{LP}$  is thus obtained. It is the protein receptor that determines the specificity of toxin binding as well as the intracellular compartment where the toxin is internalized. At the neuromuscular junction the protein receptor is responsible for the different trafficking of tetanus neurotoxin and of the botulinum neurotoxins.

is the product of the association constants of the toxin to the protein receptor and to acidic lipids (Fig. 3). Nishiki *et al.* (1994) have recently reported evidence in favour of such a mode of binding for BoNT/B and have identified the protein receptor as synaptotagmin, a transmembrane protein of small synaptic vesicles. The protein receptor of the other clostridial neurotoxins is not yet known: in any case it must be a protein of fundamental physiological importance for the neuronal

physiology. This protein cannot be deleted or altered under the selective evolutionary pressure exerted by the neurotoxins. In this respect, bacterial toxins can be compared to viruses, small pathogens that parasitize cell surface proteins to enter cells. Viruses are known to use as cell receptors a variety of plasma membrane proteins, including growth factors receptors, transporters or proteins involved in cell-cell interactions (Ross, 1990; Vile & Weiss, 1991).

The region(s) of the neurotoxins responsible for the neurospecific binding have not been mapped. Available evidence indicate that the H<sub>C</sub> domain plays a major role (Helting & Zwisler, 1977; Morris *et al.* 1980; Shone *et al.* 1985; Weller *et al.* 1986; Kozaki *et al.* 1989).

To reach its final site of action, TeNT has to enter inside two different neurons: a peripheral neuron and the inhibitory interneuron of the spinal cord (Fig. 4). There is evidence that TeNT binding to peripheral and central presynaptic terminals is different: (a) cats and dogs are highly resistant to TeNT administered peripherally, but very sensitive to the toxin injected directly in the spinal cord (Shumaker *et al.* 1939); (b) Takano *et al.* (1989) have shown that the L-H<sub>N</sub> fragment of TeNT does not cause tetanus when injected in the leg muscles of cats, but induces a spastic paralysis after direct injection in the spinal cord. As mentioned above (section 2), it is likely that TeNT reaches high concentrations in the motoneuron-inhibitory interneuron synaptic cleft and hence polysialogangliosides can mediate TeNT entry in CNS neurons. More generally, acidic lipids may be sufficient to bind and guide the internalization of TeNT, when the toxin is present in large amounts or when the fragment L-H<sub>N</sub> is used. Experiments with lipid monolayers have clearly documented the ability of 10<sup>-8</sup> M TeNT to interact with acidic lipids (Schiavo *et al.* 1991a). Similar concentrations are routinely used with cells in culture or in experiments of induction of a flaccid paralysis in mice treated with a thousand fold excess above the mouse lethal dose (Matsuda *et al.* 1982). On the contrary, in clinical tetanus and botulism, at the periphery, the CNTs are present at sub-picomolar concentrations, and hence a protein receptor must be involved in order to increase affinity and provide specificity of binding. The protein receptor of TeNT is responsible for its inclusion in a vesicle that moves in a retrograde direction, while the protein receptors of the BoNTs drive these neurotoxins inside small synaptic vesicles that acidify within at the NMJ (see Fig. 4). The TeNT carrying vesicle reaches the cell body and fuses with the Golgi or trans Golgi network or another membrane compartment where it is sorted from the protein receptor. TeNT exits inside a vesicle that moves to dendritic terminals and releases the toxin, which then equilibrates between the pre- and post-synaptic membranes. Such a model accounts for all available experimental evidence and is also in agreement with the failure to identify a TeNT protein receptor in the plasmalemma of CNS neurons using all the biochemical techniques which have identified the receptors of other protein ligands (Schiavo *et al.* unpublished work).

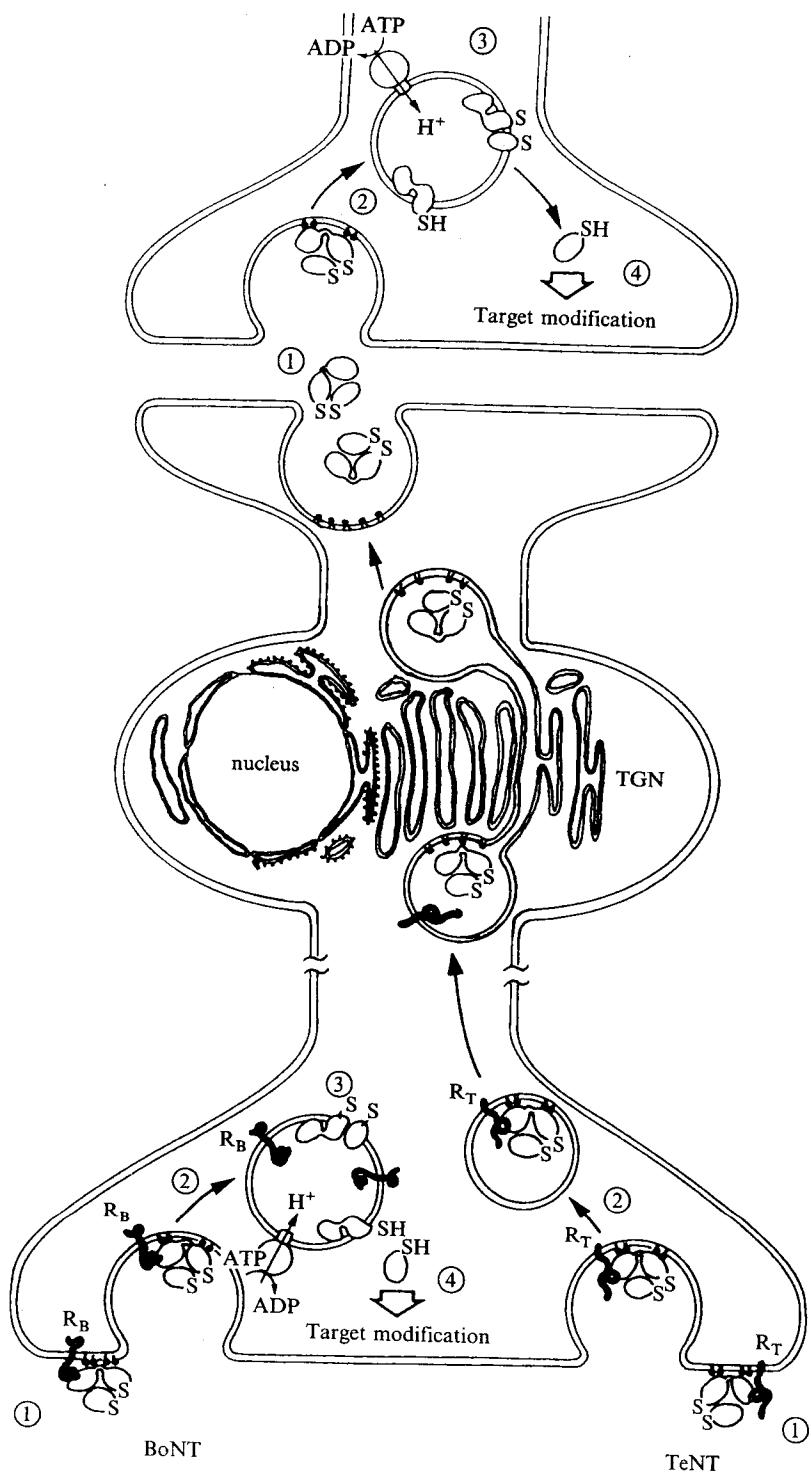


Fig. 4. Tetanus and botulinum neurotoxins intoxicate central and peripheral neurons respectively, via a four-step mechanism. TeNT and BoNTs bind to the neuromuscular junction as depicted in fig. 3. The protein receptor  $R_B$  is a synaptic vesicle protein which exposes its toxin binding domain upon fusion of the vesicle with the presynaptic membrane.

### 5. INTERNALIZATION

Since it is the L chain of TeNT and BoNTs that are responsible for the cytosolic activity of the CNTs (see section 7), at least this domain of the toxin molecule must reach the cell cytosol. There is clear pharmacologic and morphologic evidence that the CNTs do not enter the cell directly from the plasma membrane. They are endocytosed inside acidic cellular compartments. In fact, electron microscopic investigations show that, after binding, the CNTs enter the lumen of vesicular structures in a temperature and energy dependent process (Montesano *et al.* 1982; Dolly *et al.* 1984; Critchley *et al.* 1985; Black & Dolly, 1986*a, b*; Staub *et al.* 1986; Parton *et al.* 1987). Montesano *et al.* (1982) found TeNT inside non-clathrin coated vesicles, but their study was performed on liver cells exposed to very large concentrations of TeNT. Parton *et al.* (1987) examined the internalization of gold-labeled TeNT into spinal cord neurons and found it inside a variety of vesicular structures: a minority of TeNT was also found in the lumen of small synaptic vesicles.

Though the precise nature of the intracellular toxin-containing compartment remains to be determined, there is considerable evidence that its luminal pH is acidic and that this passage through a acidic compartment is required for the intoxication to occur. In fact, agents that interfere with intracellular vesicular acidification inhibit the toxicity of these neurotoxins (Simpson 1982, 1983; Simpson *et al.* 1994; Williamson & Neale, 1994).

On the basis of the fact that nerve stimulation facilitates intoxication (Ponomarev, 1928; Kryzhanovsky, 1958; Hughes & Whaler, 1962; Wellhoner *et al.* 1973; Habermann *et al.* 1980), we suggest that TeNT and BoNTs are taken up at the NMJ inside small synaptic vesicles or other vesicles whose recycling is linked to synaptic activity. The CNTs bind the intraluminal portion of a vesicle protein while the lumen is in contact with the external medium. Uptake of antibodies, which have the same molecular mass of the neurotoxins, following vesicle exocytosis and endocytosis in hippocampal neurons has been clearly documented (Matteoli *et al.* 1992). This suggestion is supported by the finding that BoNT/B binds to synaptotagmin in the presence of polysialogangliosides (Nishiki *et al.* 1994). However, if both TeNT and BoNT are inside small synaptic vesicles at peripheric synaptic terminals, it remains to be explained why BoNT

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BoNTs bind and are internalized following vesicle endocytosis (step 2). The lumen of the synaptic vesicle becomes acidic following the operation of the vacuolar ATPase proton pump. This induces a structural transition of BoNT, which adopts a 'acid structure' able to insert in the vesicle membrane and to translocate the L chain in the cytosol (step 3). Inside the neuron cytosol, L blocks neuroexocytosis via selective zinc-dependent proteolysis of a protein component of the neuroexocytosis apparatus (step 4). TeNT binds to R<sub>T</sub>, a protein receptor that mediates its internalization inside cell vesicles that are transported retroaxonally up to the cell body. Here these vesicles fuse with the TGN or an equivalent compartment, where sorting of R<sub>T</sub> from the toxin takes place. TeNT exits from the intermediate compartment inside a vesicle which moves to dendrites and it is discharged in the intersynaptic space between the motoneuron and the spinal cord inhibitory interneuron. TeNT is internalized inside the inhibitory interneuron, enters the cytosol, as BoNTs do at the neuromuscular junction, and inhibits the release of GABA and glycine.

escapes from the lumen into the cytosol to block ACh release, while TeNT remains inside the vesicle and it is delivered to the CNS.

#### 6. TRANSLOCATION INTO THE NEURONAL CYTOSOL

To reach the cytosol, the CNTs contained inside vesicles of the presynaptic terminal must cross the hydrophobic barrier of the vesicle membrane. The different trafficking of TeNT and BoNT at the NMJ clearly indicates that internalization is not necessarily followed by membrane translocation in the cytosol.

As mentioned above, there is indirect, but compelling evidence that TeNT and BoNTs have to pass through a low pH step for nerve intoxication to occur (Simpson 1982, 1983; Simpson *et al.* 1994; Adler *et al.* 1994; Williamson & Neale, 1994; Matteoli *et al.*, submitted). It is also clear that acid pH does not induce a direct activation of the toxin via a structural change, since the introduction of the L chain in the cytosol is sufficient to block exocytosis (Penner *et al.* 1986; Poulain *et al.* 1988; Anhert-Hilger *et al.* 1989*b*; Bittner *et al.* 1989*a, b*; Mochida *et al.* 1989; Weller *et al.* 1991). Hence, low pH is instrumental in the process of membrane translocation of the L chain from the vesicle lumen into the cytosol (Fig. 4). In this respect, TeNT and BoNTs resemble the other bacterial protein toxins with a three-domain structure (Menestrina *et al.* 1994; Montecucco *et al.* 1994).

Membrane interaction of CNTs has been studied mainly with model membrane systems and little data have been obtained *in vivo*. Available evidence indicates that at low pH TeNT and BoNTs undergo a conformational change from a water soluble 'neutral' form to an 'acid' form, characterized by the exposure of hydrophobic segments. This hydrophobicity enables the penetration of both the H and L chains in the hydrocarbon core of the lipid bilayer (Boquet & Duflot, 1982; Boquet *et al.* 1984; Cabiaux *et al.* 1985; Roa & Boquet, 1985; Montecucco *et al.* 1986, 1989; Menestrina *et al.* 1989; Schiavo *et al.* 1991*a*). Following this low pH induced membrane insertion, TeNT and BoNTs form ion channels in planar lipid bilayers (Boquet & Duflot, 1982; Hoch *et al.* 1985; Donovan & Middlebrook, 1986; Shone *et al.* 1987; Gambale & Montal, 1988; Blaustein *et al.* 1989; Menestrina *et al.* 1989; Rauch *et al.* 1990; Schmid *et al.* 1993). These ion-conducting channels are cation-selective, have conductances of few tens of pS and are permeable to molecules smaller than 700 daltons. There is evidence that channels are formed by toxin oligomerization of the H<sub>N</sub> domain of four toxin molecules (Donovan & Middlebrook, 1986; Shone *et al.* 1987; Menestrina *et al.* 1989; Schmid *et al.* 1993). The H<sub>N</sub> domain of CNTs includes several segments which may form amphipathic  $\alpha$ -helices and thus may be candidates for channel formation (Beise *et al.* 1994; Lebeda & Olson, 1994). Peptides corresponding to segment 668–690 of TeNT (GVVLLLEYIPEITLPVIAALSIA) and segment 659–681 of BoNT/A (GAVILLEFIPEIAIPVLGTFALV), predicted to be amphipathic  $\alpha$ -helices, form channels with properties similar to those of the intact toxin molecule (Montal *et al.* 1992). On this basis, it was proposed that the channel

is formed by a toxin tetramer that brings four amphipatic helices in proximity, with the carboxylates of the two Glu residues of the segment pointing inside the channel (Montal *et al.* 1992). This is in agreement with visualization by three-dimensional image reconstruction of the channel formed by BoNT/B in phospholipid bilayers (Schmid *et al.* 1993). Recently, Beise *et al.* (1994) were able to record with patch-clamp techniques single channels formed by TeNT in spinal cord neurons. These channels appear with high frequency at pH 5.0, but not at neutral pH, and have single channel conductance of 45 pS and are non selective for ions, including  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ba}^{2+}$  and  $\text{Cl}^-$ . Hence, TeNT, like other three-domain bacterial protein toxins, forms ion-conducting channels on cells at acidic pH values (Sandvig & Olsnes, 1988; Papini *et al.* 1988; Milne & Collier, 1993; Beise *et al.* 1994). There is a general consensus that these toxin channels are related to the process of translocation of the enzymic domain across the vesicle membrane into the nerve cytosol. However, there are different views on how this process may take place (Fig. 5).

According to a first hypothesis, the L chain unfolds at low pH and enters a transmembrane pore formed by an oligomer of the H chain. The L chains refolds within the neutral cytosolic medium and it is released from the vesicle by reduction of the interchain disulfide bond (Hoch *et al.* 1985) (see panel A of Fig. 5). In this 'tunnel' model, the formation of a transmembrane ion-conducting pore is a pre-requisite for translocation. However, this model leaves unexplained two experimental results: (a) the membrane inserted L chain of TeNT is in contact with the fatty acid chains of phospholipids, i.e. it is not shielded from lipids inside the H chain tunnel (Montecucco *et al.* 1986, 1989); (b) though there is no direct relation between channel size and conductance, values of the order of few tens of picoSiemens do not fit with the dimensions expected for a protein channel that has to accommodate a polypeptide chain with lateral groups of different volume, charge and hydrophilicity. The protein conducting channels of the endoplasmic reticulum, *E. coli* and mitochondrial membranes, characterized in planar lipid bilayers, have a conductance of 220 picoSiemens with little or no ion selectivity (Thieffry *et al.* 1988; Simon & Blobel, 1991, 1992; Fevre *et al.* 1994). These channels are closed when plugged by a transversing polypeptide chain. A change in size or polarity of the applied voltage does not influence their conductance or gating, while it does affect CNT channels.

In another view, which overcomes these difficulties of the 'tunnel' model, the L chain translocation is proposed to take place at the lipid-protein boundary, rather than inside a proteinaceous pore (Bisson & Montecucco, 1987; Montecucco *et al.* 1991, 1994), as depicted in panel B of Fig. 5. This 'cleft' model proposes that the two toxin subunits change conformation at low pH in a concerted fashion in such a way that both of them expose hydrophobic surfaces and enter in contact with the hydrophobic core of the lipid bilayer. The 'acid' form of the toxin may have properties of a molten globule (Bychkova *et al.* 1988; van der Goot *et al.* 1991). In its 'acid' conformation, the H chain is suggested to form a transmembrane hydrophilic cleft that nestles the passage of the partially unfolded acid form of the L chain. The cytosolic neutral pH induces the L chain to refold

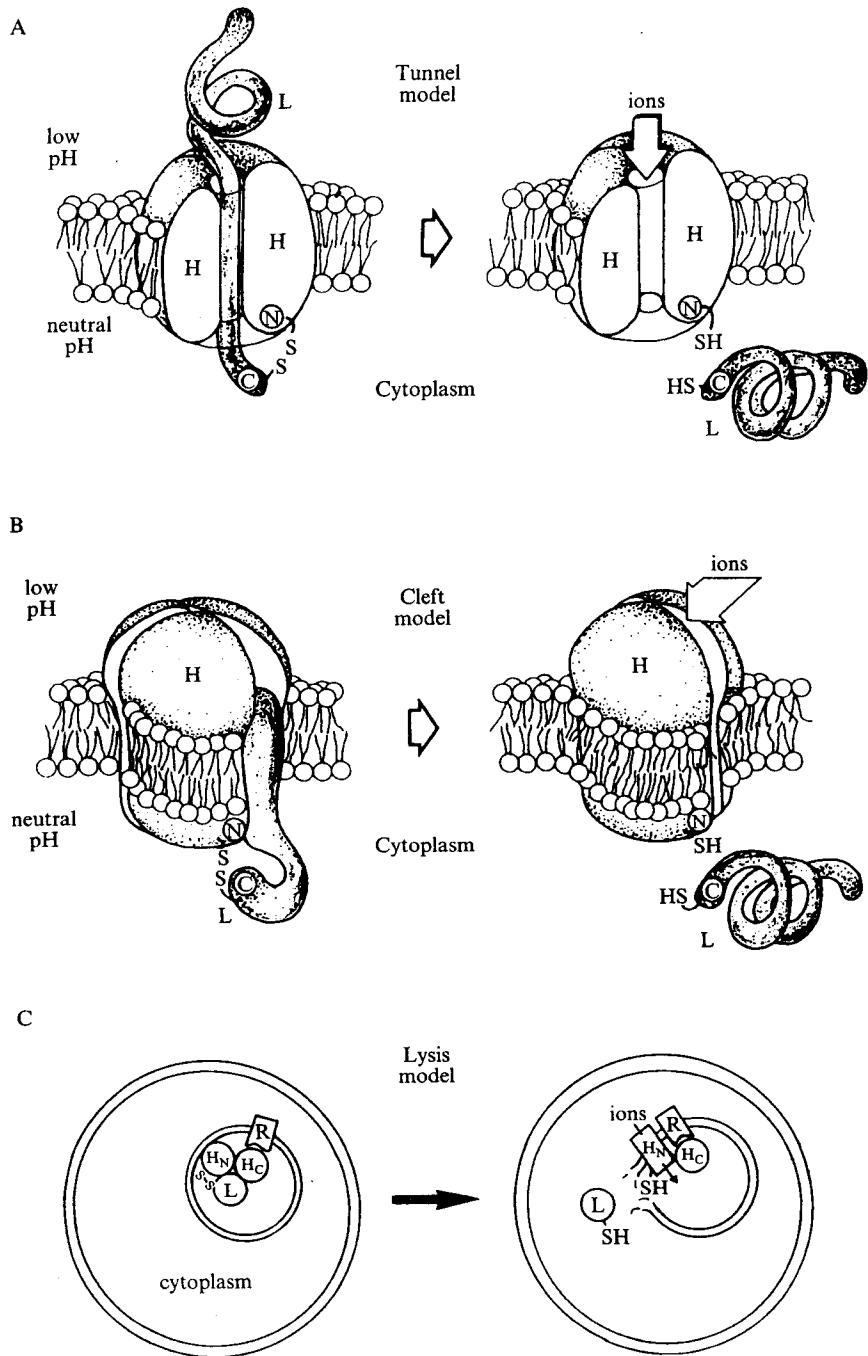


Fig. 5. Models for the membrane translocation of clostridial neurotoxins. (A) *tunnel model*: the H chain changes conformation at low pH, inserts in the lipid bilayer as an oligomer and forms a protein pore. At low pH the L chain unfolds and crosses the membrane inside the H channel. After translocation and reduction of the interchain disulfide, the L chain is released in the cytosol where it refolds and displays its zinc-endopeptidase activity. During membrane translocation, the L chain does not enter in contact with lipids. The H channel can now translocate ions. (B) *cleft model*: at low pH the H and L chains change

and to regain its water soluble 'neutral' conformation. While the L chain is leaving the vesicle membrane, the transmembrane hydrophilic cleft of the H chain tightens up to reduce the amount of hydrophilic protein surface exposed to the membrane hydrophobic core. However, this leaves across the membrane a peculiarly shaped channel with two rigid protein walls and a small mobile lipid seal on one side. This is proposed to be the structure responsible for the ion-conducting properties of TeNT and BoNTs. In the 'cleft' model, the ion channel is a consequence of membrane translocation, rather than a pre-requisite. Moreover, ion transport is performed by a transmembrane structure that derives from the one involved in the L chain translocation, but is not the same molecular entity, as proposed by the 'tunnel' model.

A third view that greatly simplifies the problem of toxin membrane translocation was recently discussed (Beise *et al.* 1994) (panel C of Fig. 5). It is proposed that, as the luminal pH of the vesicles is lowered by the activity of the vacuolar-type ATPase proton pump, TeNT and BoNT could penetrate the lipid bilayer and form an ion channel that grossly alters electrochemical gradients. This would eventually result in an osmotic lysis of the toxin-containing acidic vesicle, helped by lipid bilayer destabilizing effects of the toxin itself (Cabiaux *et al.* 1985). The membrane barrier is thus broken and the cargo of toxin molecules is released in the cytosol. Some experimental findings are not in favour of this third hypothesis. Diphtheria toxin forms ion channels on the plasma membrane of living cells at low pH without causing cell lysis (Sandvig & Olsnes, 1988; Papini *et al.* 1988; Alder *et al.* 1990) and also TeNT appears not to lyse neuronal cells (Beise *et al.* 1994). Clearly, more experiments are needed to clarify this mysterious third step of cell intoxication as well as the different membrane trafficking of TeNT and BoNT at the NMJ (see above section 5). A molecular understanding of such processes is a pre-requisite for the construction of vectors of biological reagents directed to neurons of the PNS and CNS, based on the H chains of the CNTs (Simpson, 1988; Johnstone *et al.* 1990; Dobrenis *et al.* 1992).

#### 7. ZINC-ENDOPEPTIDASE ACTIVITY OF CLOSTRIDIAL NEUROTOXINS

As mentioned above, the most conserved segment of the L domain of CNTs is located in the middle of the chain and contains the His-Glu-Xaa-Xaa-His zinc-binding motif of zinc-endopeptidases. This observation suggested that TeNT and the BoNTs could inhibit neuroexocytosis via a zinc-endopeptidase activity.

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conformation in a concerted way and insert together in the lipid bilayer. Hydrophobic residues of the L chain are mainly exposed to lipids, while hydrophilic residues are mainly involved in protein contacts with the H chain. When L faces the neutral pH of the cytosol, it reacquires its enzymically active conformation and leaves the membrane, after reduction of the interchain disulfide bond. The transmembrane hydrophilic cleft of the H chain is suggested to reduce its size in order to reduce its interaction with lipids, but can still mediate the translocation of small ions across the membrane. (C) *vesicle lysis model*: at low pH the toxin inserts in the lipid bilayer and forms an ion channel that leads to an osmotic unbalance between the vesicle lumen and the cytosol. As a result, the vesicle bursts and releases its toxic content in the cytosol.

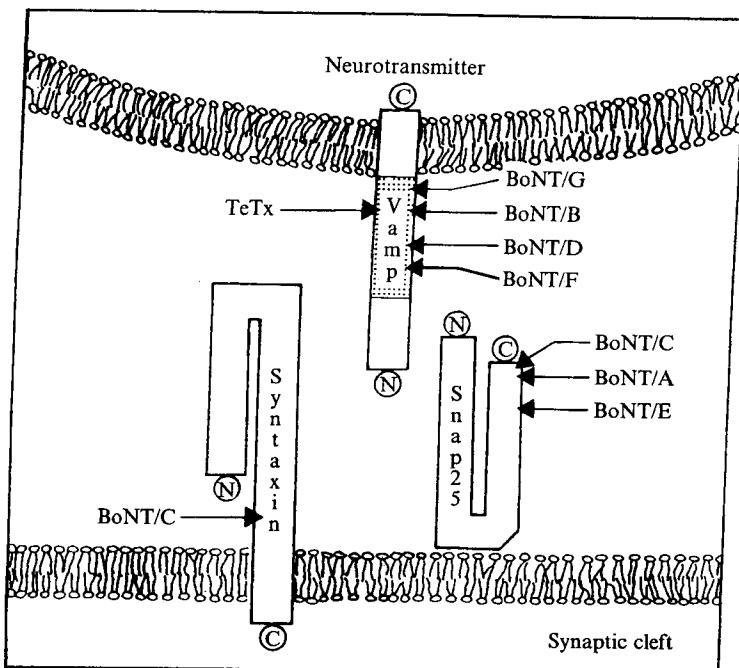


Fig. 6. Sub-cellular localization of VAMP, SNAP-25 and syntaxin and sites of cleavage of the clostridial neurotoxins. VAMP is bound to the synaptic vesicle membrane, whereas SNAP-25 and syntaxin are bound to the target membrane. TeNT and BoNT/B, /C, /D, /F and /G act on the conserved central portion of VAMP (dotted area) and release the amino-terminal part of VAMP in the cytosol. BoNT/A and /E cleave SNAP-25 at the carboxyl terminus with the release of nine and twenty six residues respectively. Also BoNT/C was very recently found to cleave SNAP-25 at the CCOH-terminus (our unpublished data). BoNT/C cleaves syntaxin at a single site near the cytosolic membrane surface. Each neurotoxin cleaves a different peptide bond. The action of TeNT and BoNT/B, /C, /D, /F and /G causes the release of a large portion of the cytosolic domain of VAMP and syntaxin. At variance, only a small segment of SNAP-25 is released by the selective proteolysis of BoNT/A, /C and /E.

However, m-RNAs encoding for TeNT L chain mutated at the histidines of the motif did inhibit ACh release when injected into *Aplysia* neurons (Binz *et al.* 1992). Since, it is well established that corresponding mutations in a variety of well characterized zinc-endopeptidases abolish enzymic activity (Barrett, 1995), this report favoured the possibility that another catalytic activity was displayed by the CNTs in the cytosol. However, injection of the apo-TeNT L chain in *Aplysia* neurons indicated the essential role of the metal atom in toxin activity (Schiavo *et al.* 1992 *a, c*). Moreover, phosphoramidon, known to be a very specific, though not general, inhibitor of zinc-endopeptidases, also inhibited the TeNT-induced blockade of ACh release (Schiavo *et al.* 1992 *a*). This was the first clear evidence that the L chain of TeNT was acting inside nerves via a metalloprotease activity.

Since neurotransmitters at the presynaptic terminals are mainly contained inside SSVs, these latter organelles were the first candidate targets of the neurotoxin proteolytic action. It was rapidly demonstrated that protease-free

Table 2. *Tetanus and botulism neurotoxins (target and peptide bond specificities)*

Toxin type	Target	Peptide bond cleaved P <sub>3</sub> - P <sub>2</sub> - P <sub>1</sub> -P <sub>1'</sub> - P <sub>2'</sub> - P <sub>3'</sub>
TeNT	VAMP	Ala- Ser- Gln- Phe- Glu- Thr
BoNT/A	SNAP-25	Ala- Asn- Gln- Arg- Ala- Thr
BoNT/B	VAMP	Ala- Ser- Gln- Phe- Glu- Thr
BoNT/C <sup>a</sup>	syntaxin	Thr- Lys- Lys- Ala- Val- Lys
BoNT/D	VAMP	Asp- Gln- Lys- Leu- Ser- Glu
BoNT/E	SNAP-25	Ile- Asp- Arg- Ile- Met- Glu
BoNT/F	VAMP	Arg- Asp- Gln- Lys- Leu- Ser
BoNT/G	VAMP	Thr- Ser- Ala- Ala- Lys- Leu

<sup>a</sup> In neurons intoxicated with BoNT/C also SNAP-25 is cleaved (Osen-Sand *et al.* submitted).

preparations of TeNT and BoNT/B, /D, /F and /G cleave VAMP, also called synaptobrevin, a membrane protein of synaptic vesicles (Schiavo *et al.* 1992a, c, 1993b, c, 1994; Yamasaki *et al.* 1994a, b; Schiavo & Montecucco, 1995). At variance, BoNT/A, /C and /E act on proteins of the presynaptic membrane: BoNT/A and /E cleave SNAP-25, while serotype C cleaves syntaxin, in addition to SNAP-25 (Blasi *et al.* 1993a, b; Schiavo *et al.* 1993c, d, 1995a; Binz *et al.* 1994; Osen-Sand *et al.*, in preparation). Fig. 6 shows schematically the localization of VAMP, SNAP-25 and syntaxin and the position of the cleavage sites of the various CNTs. Recombinant VAMP, SNAP-25 and syntaxin are cleaved at the same peptide bonds and with the same rate as the corresponding cellular proteins thus indicating that no additional endogenous factors are involved in the proteolytic activity of the CNTs.

The peptide bond hydrolysed by each neurotoxin has been identified (Table 2). Apart from TeNT and BoNT/B, each one of the different CNTs catalyses the hydrolysis of a different peptide bond. Moreover, the amino acid residues flanking the cleavage sites also differ in terms of charge, hydrophilicity/hydrophobicity and volume. Hence, the active sites of these metalloproteinases must all differ in their spatial organization, in order to accommodate and hydrolyse such different peptide bonds.

Many other neuronal and non-neuronal proteins have been assayed as possible proteolytic substrates with negative results. The finding that VAMP, SNAP-25 and syntaxin are the only known substrates of neurotoxins, which cause a prolonged inhibition of neurotransmitter release, clearly demonstrates that these three proteins play a central role in neuroexocytosis.

#### 8. VAMP, SNAP-25 AND SYNTAXIN

VAMP was first cloned from a *Torpedo* electric organ library (Trimble *et al.* 1988), and then from rat, bovine, human, *Drosophila*, yeast, squid and *Aplysia* (Elferink

*et al.* 1989; Sudhof *et al.*, 1989; Archer *et al.* 1990; Di Antonio *et al.* 1993; Protopopov *et al.* 1993; Hunt *et al.* 1994; Yamasaki *et al.* 1994a; Sweeney *et al.* 1995).

VAMP is about 120 residues long and the exact number of residues depends on source and isotype. As shown in Fig. 6, a short carboxyl-terminal segment is inside the vesicle lumen (Trimble *et al.* 1988; Baumert *et al.* 1989), whereas about five sixths of the molecule are exposed to the cytosol. The thirty-residue long amino-terminus diverges considerably among species and isoforms. It has a high proline content and appears to belong to a group of cytosolic domains involved in low affinity, but specific protein-protein interactions (Williamson, 1994). The central part of VAMP (residues 30–96, dotted in Fig. 6) is highly conserved, is rich in charged and hydrophilic residues, and includes all the known cleavage sites of the neurotoxins. On the synaptic vesicle membrane, VAMP is associated with synaptophysin (Calakos & Scheller, 1994; Washbourne *et al.* 1995), a protein able to form oligomers endowed with channel properties which may be directly involved in neurotransmitter release (Thomas *et al.* 1988).

Three isoforms of VAMP have been so far identified: VAMP-1, VAMP-2 and cellubrevin (Elferink *et al.* 1989; Archer *et al.* 1990; McMahon *et al.* 1993). Though data are far from complete, available evidence indicate that VAMP is present in all vertebrate tissues (Baumert *et al.* 1989; Corley-Cain *et al.* 1992; MacMahon *et al.* 1993; Chin *et al.* 1994; Gaisano *et al.* 1994; Hohne-Zell *et al.* 1994; Ralston *et al.* 1994; Jacobsson *et al.* 1994; Papini *et al.* 1995; Regazzi *et al.*, 1995; Sweeney *et al.* 1995; Rossetto *et al.*, submitted). What varies is the distribution of VAMP-1 and -2 among the various cell types (Rossetto *et al.*, submitted). The precise functional implication of this differential distribution of VAMP isoforms is unknown, but clearly signifies that VAMP is of central importance in many events of vesicle fusion, and not only in neuroexocytosis. Moreover, these recent findings indicate that the neurospecific action of TeNT and BoNTs is due to cell surface receptors able to mediate their cellular uptake, and not to lack of cleavable targets.

VAMP-1 of chickens and rats are not cleaved by CNTs (Schiavo *et al.* 1992c; Schiavo *et al.*, unpublished results). These VAMPs have a Val residue in place of the Gln residue present in human and mouse VAMP-1 at the cleavage site of TeNT and BoNT/B. The structural basis of this lack of proteolysis is not known. However, this region of VAMP is predicted to bend at the Gln-Phe peptide bond. Since Val is a helix-inducing residue and proteases are known to cleave very poorly alpha-helical regions, a different structure of rat and chicken VAMP-1 may account for this effect. This single amino acid replacement at the cleavage site has been proposed to be associated with the resistance of rats and chickens, and with the sensitivity of humans and mice, to tetanus and to type B botulism (Patarnello *et al.* 1993). The differential susceptibility to TeNT and BoNT/B proteolysis of rat VAMP-1 and -2 has been used to identify the VAMP isoforms (Braun *et al.* 1994; Steinhardt *et al.* 1994.). At variance, BoNT/D, /F and /G cleave both VAMP-1 and -2 with similar rates.

SNAP-25 is a 206 residue-long protein lacking a transmembrane segment and

is bound to the cytosolic surface of the nerve plasmalemma, possibly via palmitoylation of four cysteines, located at the center of the molecule (Oyler *et al.* 1989; Hess *et al.* 1992). SNAP-25 is required for axonal growth during development and possibly nerve terminal plasticity in the mature nervous system (Osen-Sand *et al.*, 1993). The protein is highly conserved from *Drosophila* to mammals (Bark, 1993; Risinger & Larhammar, 1993; Risinger *et al.* 1993). In humans, there are two isoforms differentially expressed during development: isotype a is constitutively expressed beginning at the embryo stage, whereas isoform b appears at birth and is the predominant form in adult life (Bark & Wilson, 1994). The tissue distribution of SNAP-25 is less known than that of VAMP. However, its presence in pancreatic cells (Jacobsson *et al.* 1994; Sadoul *et al.* 1995) may indicate that it is also widely expressed outside the nervous tissue. Two SNAP-25 related proteins have been recently cloned from yeast and they lack the cysteine quartet, possibly indicating that palmitoylation may play a functional, rather than a structural, role in nerve cells (Brennwald *et al.* 1994).

Syntaxin is also located on the cytosolic surface of the nerve plasmalemma (Bennett *et al.* 1992; Inoue *et al.* 1992). It is membrane-anchored via a carboxy-terminal segment and most of its molecular mass is exposed to the cytosol (Fig. 6). Syntaxin colocalizes with calcium channels at the active zones of the presynaptic membrane, where neurotransmitter release takes place (Bennett *et al.* 1992). Syntaxin also interacts with synaptotagmin, a protein of the SSV membrane, that forms a functional bridge between the plasmalemma and the vesicles (Bennett & Scheller, 1994). Two isoforms of slightly different length (285 and 288 residues) have been identified in nerve cells (isotypes 1A and 1B) and other isoforms are present in other tissues (2, 3, 4 and 5) (Bennett *et al.* 1993). BoNT/C cleaves syntaxin only when inserted in lipid bilayers containing acidic lipids and only isoforms 1A, 1B, 2, 3 are BoNT/C sensitive (Blasi *et al.* 1993*b*; Schiavo *et al.* 1995*a*).

Evidence that TeNT and BoNTs block neuroexocytosis via the specific cleavage of VAMP, SNAP-25 and syntaxin are growing:

- (a) the intracellular activity of the toxins is inhibited by specific inhibitors of zinc-endopeptidases such as phosphoramidon and captopril (Schiavo *et al.* 1992*a*; de Paiva *et al.* 1993*b*; Dayanithi *et al.* 1994; Poulain *et al.* unpublished results).
- (b) VAMP or SNAP-25 or syntaxin are cleaved in synaptosomes and PC12 cells intoxicated with TeNT or BoNTs (Link *et al.* 1992; Blasi *et al.* 1993*a, b*; Schiavo *et al.* 1993*b*; Adler *et al.* 1994; Hohne-Zell *et al.* 1994; Papini *et al.* 1995).
- (c) peptides spanning the cleavage site of VAMP inhibit TeNT and BoNT/B in *Aplysia* neurons or chromaffin cells (Schiavo *et al.* 1992*b*; Dayanithi *et al.* 1994).
- (d) an antibody highly specific for VAMP prevents the inhibition of neurotransmitter release in *Aplysia* neurons induced by TeNT and BoNT/B, but not that caused by BoNT/A (Poulain *et al.* 1993).
- (e) an antibody against the zinc-binding segment inhibits the activity of TeNT and BoNT/A in chromaffin cells (Bartels *et al.* 1994).
- (f) the inhibition of neurotransmitter release in cultured cortical neurons is

paralleled by VAMP, or SNAP-25 or syntaxin proteolysis assayed with specific antibodies (Osen-Sand et al., submitted).

These results do not exclude that CNTs may act on other substrates, but clearly indicate that VAMP, SNAP-25 and syntaxin are major targets of the proteolytic activity of clostridial neurotoxins. On this basis, CNTs are increasingly used to probe the involvement of these three proteins in a variety of cell events (Steinhardt et al. 1994; Braun et al. 1994; Gaisano et al. 1994; Hohne-Zell et al. 1994; Ikonen et al. 1995; Papini et al. 1995; Regazzi et al. 1995; Sadoul et al. 1995).

#### 9. TARGET RECOGNITION

More than hundred peptides were also tested as possible *in vitro* proteolytic substrates. Some of them were designed on the basis of the known protein sequence around the cleavage sites of the various toxins. Invariably, none of these peptides was cleaved (Schiavo et al. 1992a; Shone et al. 1993; Cornille et al. 1994; Shone & Roberts, 1994; Yamasaki et al. 1994b). On the other hand, peptides encompassing the cleavage sites do bind to the respective toxins and inhibit their proteolytic activity (Schiavo et al. 1992c, 1993a, 1994). Shone et al. (1993) found that BoNT/B proteolysis of VAMP peptides becomes appreciable with forty-residue long peptides and that maximal cleavage rates were only observed with the 33–96 VAMP segment. Interestingly, Yamasaki et al. (1994b) found that BoNT/F and /D appear to require peptides more extended at their amino-terminal than those hydrolysed by TeNT and BoNT/B. A requirement for long peptides is also shown by BoNT/A and /E, which hydrolyse the SNAP-25 fragment 136–206 (Binz et al. 1994). Another peculiarity of these metalloproteinases is that, within VAMP or SNAP-25 or syntaxin, they hydrolyse only one out of several identical peptide bonds present in the target protein sequence. These features indicate that TeNT and BoNTs are proteinases that recognize the tertiary structure of their targets and that the cleaved peptide bond with flanking residues is not the sole determinant of the very specific recognition of VAMP, SNAP-25 and syntaxin (Schiavo et al. 1993a; Montecucco & Schiavo, 1993, 1994).

As mentioned above, the L chains have strongly similar primary and secondary structures, thus indicating that they derive from a common ancestral metalloprotease. This close similarity suggested that the three targets could share a region involved in toxin binding. An inspection of the sequences of VAMP, SNAP-25 and syntaxin, revealed that these three proteins share a short motif (Fig. 7 A and B), which is comprised within regions predicted to adopt an  $\alpha$ -helical conformation (Rossetto et al., 1994). The motif, plotted as an  $\alpha$ -helix, shows a face with three negative charges contiguous to a face formed by hydrophobic residues (Fig. 7 C and D). This motif is present in all VAMP, SNAP-25 and syntaxin isoforms known to be expressed in the nervous tissue of animals sensitive to the neurotoxins. Variations are present in VAMP and syntaxin of *Drosophila* and yeast, species which are resistant to these neurotoxins, and in syntaxin isoforms known not to be involved in exocytosis. There are two copies of the motif in VAMP, four copies in SNAP-25 and two copies in syntaxin (Fig. 7 A). Peptides

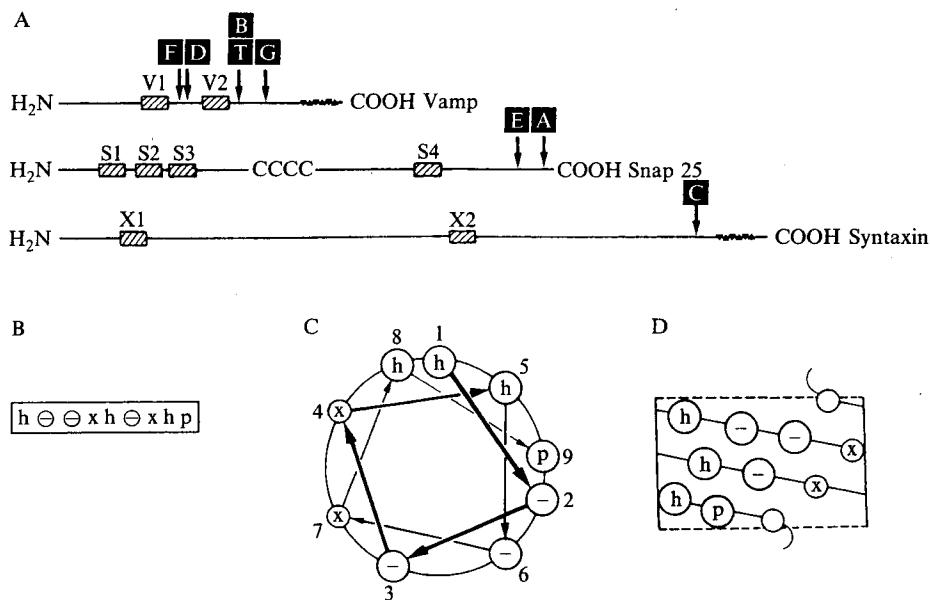


Fig. 7. Neurotoxin recognition motif of VAMP, SNAP-25 and syntaxin. (A) hatched boxes indicate the presence and positions of a motif common to the three targets of clostridial neurotoxins; (B) shows the motif composed of h (hydrophobic residue), – (Asp or Glu residue), p (polar residue) and x (any residue). The motif is included in the three proteins within segments predicted to adopt an  $\alpha$ -helical conformation. (C) and (D) show a top and a later view of the motif, respectively.

corresponding to the specific sequences of the motifs of each of the three neurotoxin target proteins inhibit *in vitro* and *in vivo* the activity of the neurotoxins, irrespectively of their origin and the neurotoxin type used. Antibodies raised versus these peptides cross-react among the three target proteins (Rossetto *et al.* 1994; Pellizzari *et al.* in preparation). These results indicate that the motif is exposed on the protein surface and adopts a similar configuration in each of the three neurotoxin targets. As predicted from these findings, the VAMP-specific, the SNAP-25-specific and the syntaxin-specific neurotoxin types cross-inhibit each other, namely they are able to compete for the same binding site, though are unable to cleave other targets than their specific one (Rossetto *et al.* 1994; Pellizzari *et al.* in preparation). Taken together, these results indicate that TeNT and BoNTs recognize their protein substrates via two regions that interact with: (a) a segment that includes the peptide bond to be cleaved and (b) another segment closely similar in VAMP, SNAP-25 and syntaxin, which accounts for antibody cross-reactivity and cross-inhibition of the different neurotoxin types. The relative contribution of segment (a) and (b) to the specificity and strength of neurotoxin binding remains to be determined. It can be anticipated that hydrolysis of region (a) leaves the toxin bound to its substrate only via its interaction with (b) with a large decrease in binding affinity, which is expected to lead to a rapid release of the hydrolysed substrate. Such a mode of

binding accounts for the high concentrations of peptides needed to inhibit the toxin protease activity (Rossetto et al., 1994). A 'two arms' model also explains the available data on the minimal size of the target still cleaved by the neurotoxins (Shone et al. 1993; Binz et al. 1994; Yamasaki et al. 1994b; Shone & Roberts, 1994), because, in order to be cleaved, a peptide must include both (a) and (b). Moreover, this model explains why the n-syb isoform of *Drosophila* VAMP with two non-conservative mutations in the (b) region and identical site of cleavage, is not proteolysed by TeNT (Sweeney et al. 1995). An analysis of recent results (Shone et al. 1993; Binz et al. 1994; Yamasaki et al. 1994b; Shone & Roberts, 1994; Schiavo et al. 1995a; Sweeney et al. 1995) indicates that V<sub>1</sub> is involved in binding BoNT/D and /F, V<sub>2</sub> is involved in binding TeNT, BoNT/B and /G, S<sub>4</sub> is involved in binding BoNT/A, and /E and S<sub>4</sub> and X<sub>2</sub> are the sites of BoNT/C binding (Pellizzari et al. in preparation). Mutation of the various residues of the motif are under way and preliminary results indicate that the negative charges of the carboxylate residues are essential for substrate recognition (Pellizzari et al. in preparation).

The regions of TeNT and BoNTs involved in substrate binding are unknown. It is tempting to suggest that the strongly conserved one hundred-residue long amino-terminal region is implicated. Removal of more than eight residues from the amino-terminus leads to complete loss of activity (Kurazono et al. 1992). This region includes a segment (80–100) predicted to adopt an  $\alpha$ -helical structure (Lebeda & Olson, 1994). It is noteworthy, that this segment is rich in positively charged residues that would lie on the same face of the helix and could interact electrostatically with the negative charges of the motif.

#### 10. THE NEUROEXOCYTOSIS APPARATUS AND THE CLOSTRIDIAL NEUROTOXINS

Recently, Söllner et al. (1993a, b) have shown that VAMP, SNAP-25 and syntaxin, together with a group of cytosolic proteins (NSF,  $\alpha$ -SNAP,  $\beta$ -SNAP,  $\gamma$ -SNAP), form a 20S multi-protein complex, proposed to mediate vesicle docking and fusion within the cell (Rothman & Warren, 1994; Rothman, 1994). Moreover, there is evidence that synaptotagmin and synaptophysin, two integral proteins of SSV, rab<sub>3</sub>A, a small GTP-binding protein, and MUNC-18 are also involved in the process (Sudhof & Jahn, 1991; Bennett & Scheller, 1994). In addition to exocytosis, vesicle targeting and fusion are the basis of many events of cell membrane trafficking, including endocytosis, protein maturation and export and formation and control of cell organelles (Simons & Zerial, 1993; Ferro-Novick & Jahn, 1994; Rothman & Warren, 1994; Rothman, 1994). It was proposed that specificity of the delivery of the cargo of each vesicle type is ensured by the recognition between a vesicle receptor protein (termed v-SNARE) and a complementary receptor protein located on the target membrane (t-SNARE). It was also proposed that the v-SNARE role may be played by VAMP and the t-SNARE role by syntaxin and that specific VAMP and syntaxin isoform couples control each vesicle docking event within one cell (Söllner et al. 1993a; Rothman,

1994; Rothman & Warren, 1994). The finding that the proteins putatively involved in the process are present and conserved in all living organisms strongly support this model.

It was recently shown that SNAP-25 and syntaxin form a 1:1 complex, able to bind one molecule of VAMP, and that this trimer is SDS stable (Chapman *et al.* 1994; Hayashi *et al.* 1994). In the neuroexocytosis process, after SNAP-25/Syntaxin/VAMP complexation, cytosolic protein components are recruited in a reaction modulated by rab3a and the neuroexocytosis apparatus is thus assembled (O'Connor *et al.* 1994). It is likely that the NSF-mediated hydrolysis of ATP provides the energy for priming the apparatus that becomes thus ready to trigger exocytosis upon binding calcium ions to yet undefined low affinity calcium binding sites. It is not yet established if neurotransmitter release takes place via a fusion pore or via a complete membrane fusion with lipid intermixing (Burgoyne & Morgan, 1993; Fesce *et al.* 1994; Monk & Fernandez, 1994).

BoNT/A removes only nine residue from the SNAP-25 carboxy-terminus and yet this is sufficient to impair neuroexocytosis, thus indicating that this part of the molecule is essential for SNAP-25 folding. Alternatively, the carboxyl end of SNAP-25 plays an important role in the function of the neuroexocytosis complex. The fact that neuroexocytosis can be rescued by  $\alpha$ -latratoxin and by calcium in the NMJ poisoned with BoNT/A (see section 2) indicates that SNAP-25 plays a modulatory role. We have recently suggested that SNAP-25 is a calcium binding protein and that BoNT/A and BoNT/E act by lowering its calcium binding affinity (Schiavo *et al.* 1995b).

Cleavage of VAMP and of syntaxin by TeNT, BoNT/B, /D, /F, /G and /C leads to the release in the cytosol of a large part of the cytosolic target molecule. Given their proposed role as vesicular and target membrane receptors, it is expected that intoxication would impair docking. On the contrary, it appears that poisoned and electrically silent synapses show an increased number of docked vesicles, as judged from electron microscopy (Mellanby *et al.* 1988; Neale *et al.* 1989; Hunt *et al.* 1994). Thus, VAMP appears to play additional role(s) in exocytosis and possibly in vesicle re-uptake as well.

## 11. WHY THE NEUROTOXINS?

This question cannot be adequately answered with the present poor knowledge of the ecology of Clostridia in general and, in particular, of that of toxicogenic Clostridia. A successful bacterium is the one able to multiply effectively and to spread so that it is present in nature in large numbers (Mims, 1987). During evolution, the successful bacterium attains a state of 'balanced pathogenicity' such as to cause the smallest alteration of host physiology compatible with the need to enter and multiply in the body and to spread to other individuals. As noted above, NT genes are episomic. It may be assumed that chromosomal genes code for proteins essential for the bacterial life cycle, whereas episomal genes are important for related functions, such as growth and spreading under unusual environmental conditions. Living vertebrates offer within their body only very small anaerobic

habitats, where Clostridia can survive. The release of a neurotoxin that kills the animal, converts it into an anaerobic fermentor able to support the growth of billions and billions of Clostridia of endogenous as well as exogenous origin. Clostridia are known to produce a variety of hydrolases that facilitate dissolution of tissues. In this simplified view, the production of neurotoxins is functional to the creation of new habitats. During the massive Clostridia growth on the cadaver, neurotoxin genes can be exchanged via bacteriophages or conjugational plasmids or transposons. As the cadaver cannot support bacterial spreading to other hosts, Clostridia sporulates and the spores are dispersed by atmospheric agents and other natural forces. On the basis of the considerations made above, toxigenic Clostridia do not appear to be 'balanced pathogens'. However, it should be considered that killing the host is functional to the life cycle of a strictly anaerobic organism and the production of spores is essential to their survival and spreading in the environment.

The finding that CNTs are zinc-endopeptidases, specific for different proteins of the neuroexocytosis apparatus, which are cleaved at different peptide bonds, suggest a possible evolutionary origin of these neurotoxins. Clostridia produce a variety of rather non-specific proteinases that act outside cells. At a certain stage of evolution, a metalloproteinase gene has fused with another gene and has given rise to a protein able to bind and act specifically at the neuromuscular junction. Further genetic rearrangements have then lead to a toxin able to enter neurons and later evolution has then focussed the proteolytic activity of the neurotoxin on a selected protein of the multi-subunit complex that mediates neuroexocytosis. Different sites of attack of the same supramolecular structure warrant that an animal species cannot become resistant to all clostridial neurotoxins at the same time by point mutation of the site of proteolysis, as rats and chickens have done for TeNT and BoNT/B.

## 12. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The neurotoxins produced by Clostridia responsible of tetanus and botulism form a new group of zinc-endopeptidases endowed with peculiar properties. They are produced as inactive precursors which are activated by specific proteolysis followed by intracellular reduction of a single disulfide bond. Their amino acid sequence around the zinc binding motif does not resemble that of any of the various groups of zinc-endopeptidases. They act in the cell cytosol and are very specific in terms of both protein target and peptide bond cleaved. This specificity for subunits of the neuroexocytosis apparatus makes them exquisite tools in neuroscience research.

Next important steps will be the identification of the neurospecific receptors of CNTs and of their mode of internalization and membrane translocation. Another important line of research is that aimed at finding specific inhibitors of these metalloproteinases, able at the same time to cross the nerve plasmalemma. Such inhibitors would be potential therapeutic agents in the treatment of tetanus and botulism. Another result of medical importance will be the modification of BoNTs

so that their life-time inside the NMJ can be prolonged. This will extend in time the beneficial effects of the BoNT treatment of dystonia patients. These two latter points will be greatly helped by the determination of the three-dimensional structure of these neurotoxins. Highly diffracting crystals of BoNT/A and BoNT/C are already being analysed.

One final point is the evaluation of possible additional activities of these neurotoxins. Facchiano *et al.* (1993a, b) have recently reported that TeNT binds and activates synaptic transglutaminase and that synapsin is an excellent transglutaminase substrate. A transglutaminase is present on small synaptic vesicles and its activation by the toxin could cross-link synapsin to the vesicles, thus impairing their availability to release (Facchiano *et al.* 1993b; Valtorta *et al.* 1992). In addition, it was reported that BoNT/A induces a decrease of arachidonic acid release from nerve terminal membrane stores, thus affecting neuroexocytosis (Ray *et al.* 1993). The fact that the L chain of clostridial neurotoxins may possess more than one catalytic activity should be evaluated in future studies. If this is found to be the case, another marvel will be added to these proteins, in addition to their abilities to bind specifically to neuronal cell, to penetrate these cells by crossing the hydrophobic membrane barrier and to their highly specific proteolytic activity.

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